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ORIGINAL ARTICLE

Conservation and divergence of candidate class B genes in *Akebia trifoliata* (Lardizabalaceae)

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Abstract There is evidence that gene duplication and diversification within the MADS-box gene family had significant impact on floral architecture. In this study, we report the isolation of four class B homologous genes from *Akebia trifoliata*, termed *AktAP3_1*, *AktAP3_2*, *AktAP3_3*, and *AktPI*. Phylogenetic analysis indicates that the three *AktAP3* paralogs were produced by two gene duplication events and *AktAP3_2* and *AktAP3_3* are recent paralogs, which are yielded by the duplication before the origin of the genus *Akebia*. In situ hybridization demonstrates that these genes are mainly expressed in the stamens and carpels of *A. trifoliata*, but in differential patterns, similar to those in other basal eudicot and basal angiosperm species. *AktAP3_3* and *AktPI* are expressed in the developing petaloid perianth, suggesting that the petaloidy of the perianth is caused by the expression of class B genes. Reverse transcriptase polymerase chain reaction analyses indicate that these genes are expressed in both male and female flowers, but at different levels. We explore the interaction behavior of the class B proteins in the basal eudicots using yeast two-hybrid system for the first time. The AktAP3_1/2/3 proteins and the AktPI protein can form obligate heterodimers, but at different strength. From the mRNA expression and protein interaction patterns of the duplicated copies of the *AktAP3* genes, we conclude that subfunctionalization very likely contributes to the maintenance of multiple *AP3*-like gene copies in *A. trifoliata*.

Keywords *APETALA3* · Basal eudicots · Evolution · Obligate heterodimer · *PISTILLATA* · Subfunctionalization

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Introduction

Flowers are arguably the most essential key innovation of the angiosperms. The architecture of flowers is complex and diverse, especially the morphology of the perianth. In basal angiosperms, flowers generally have undifferentiated tepals or no perianth, and display a spiral arrangement of floral parts (Endress 1994; De Craene et al. 2003). In contrast, the flowers of core eudicots usually have a bipartite perianth composed of sepals and petals arranged in whorls (Endress 1992). Basal eudicots, as a transitional group connecting basal angiosperms with core eudicots, have diverse floral structures similar to either basal angiosperms or core eudicots (Albert et al. 1998; Drinnan et al. 1994; Soltis et al. 2003; Zanis et al. 2003). According to the ABC model of floral developmental control, three classes of transcription factors are responsible for the identities of four whorls of floral organs in a combinatorial manner. A-function genes are responsible for the specifica-

tion of sepals, A + B for petals, B + C for stamens, and C alone for carpels. A-function and C-function genes are antagonistically regulated (Coen and Meyerowitz 1991).

A considerable number of floral organ identity genes have been isolated from angiosperms so far. Most of them are members of the MADS-box gene family and have been grouped into different subfamilies, such as *APETALA1* (*AP1*)/*SQUAMOSA* (*SQUA*) (class A), *DEFICIENS* (*DEF*)/*GLOBOSA* (*GLO*) (class B), *AGAMOUS* (*AG*) (class C/D), and *SEPALLATA* (*SEP*) (class E) (reviewed in Becker and Theissen 2003; Ma and dePamphilis 2000; Ng and Yanofsky 2001; Riechmann and Meyerowitz 1997; Theissen et al. 1996, 2000). Among these subfamilies, the members of *AP1*/*SQUA* and *DEF*/*GLO* subfamilies are closely associated with the development of the perianth. Phylogenetic reconstructions have outlined evolutionary scenarios regarding the *AP1*/*SQUA* and *DEF*/*GLO* subfamilies and implicated that multiple duplication events within each subfamily have to be considered for elucidating the relationship between the gene evolution and floral morphological diversity (Aoki et al. 2004; Irish 2003; Kim et al. 2004b; Kramer et al. 1998; Litt and Irish 2003; Stellari et al. 2004).

Compared with the *AP1*/*SQUA* subfamily, the *DEF*/*GLO* subfamily has been studied more extensively and profoundly (reviewed in Zahn et al. 2005). It has been demonstrated that two major gene duplication events took place within this subfamily: one affecting the ancestral B gene lineage before the occurrence of extant angiosperms, producing the *DEF*/*AP3* (paleo*AP3*) and the *GLO*/*PI* lineages (Aoki et al. 2004; Kim et al. 2004b; Kramer et al. 1998); the other affecting the *DEF*/*AP3* lineage near the origin of the core eudicots, yielding the *TM6* and eu*AP3* lineages (Aoki et al. 2004; Kim et al. 2004b; Kramer et al. 1998). The eu*AP3* lineage is unique to the core eudicots and has a euAP3 motif at the end of the C-terminus. In contrast, the paleo*AP3* and *TM6* lineages share a distinguishable paleoAP3 motif at the corresponding position of the C-terminal region (Kramer et al. 1998; Vandenbussche et al. 2003). The available mutant and transgenic data of *Arabidopsis* and other core eudicot species have demonstrated that eu*AP3*-like genes have obtained a novel function contributing to the establishment of petal identity during the evolution of the core eudicots (Jack et al. 1992; Lamb and Irish 2003; Sommer et al. 1990; Vandenbussche et al. 2004; Van der Krol and Chua 1993; Van der Krol et al. 1993). Moreover, protein–protein interaction assays and molecular genetics indicate that the AP3 and PI proteins of the core eudicot species, such as *Arabidopsis*, *Antirrhinum*, and *Petunia*, perform their functions by forming obligate heterodimers (Riechmann et al. 1996; Schwarz-Sommer et al. 1992; Vandenbussche et al. 2004).

Besides these two major gene duplications, many small-scale duplications have also been documented within the *DEF*/*AP3* lineage (e.g., Di Stilio et al. 2005; Kim et al. 2005b; Kramer and Irish 2000; Kramer et al. 2003; Matsunaga et al. 2003; Stellari et al. 2004). In general, after gene duplications, some duplicated copies are retained ("survive") due to subfunctionalization and/or neofunctionalization, which are often based on the divergence of expression patterns (Duarte et al. 2006; Force et al. 1999; Lynch and Force 2000). For example, in the Ranunculaceae, three types of paleo*AP3*-like genes (*AP3-I*, *AP3-II*, and *AP3-III*) have been reported (Kramer et al. 2003). Although these paralogous genes have slightly different C-terminal regions, all of them are members of the paleo*AP3* lineage, as indicated by the presence of the typical paleo*AP3* motif. Preliminary analyses indicated that these genes exhibit differential expression patterns in the outer perianth whorls (Kramer and Irish 2000; Kramer et al. 2003). Similar situations were also found in the multiple paleo*AP3* genes from *Asarum europaeum*, a basal angiosperm species (Kramer and Irish 2000), and *Illicium floridanum*, one of the basalmost angiosperms (Kim et al. 2005b). The diversity of expression patterns of paralogous paleo*AP3* genes from the basal eudicots and basal angiosperms implies that the duplicated genes might perform their functions in different modes at the level of protein–protein interaction. Many studies have suggested that in monocots, interactions between paleoAP3 and PI proteins are quite complicated, including homodimerization, obligate heterodimerization, and facultative interaction (reviewed in Kaufmann et al. 2005). However, very little is known about the situation in basal eudicots and basal angiosperms.

Lardizabalaceae, a relatively primitive family within Ranunculales, represents one of the major clades of basal eudicots (Hoot et al. 1999; Kim et al. 2004a; Soltis et al. 2003). Among the eight genera of the Lardizabalaceae, only the flowers of *Akebia* display simple perianth morphology with three petaloid sepals (APG 2003; Qin 1997). In this study, we report three *AP3*-like genes and one *PI*-like gene from *A. trifoliata*, a species of the genus *Akebia* (APG 2003; Hoot et al. 1995; Qin 1997), and perform detailed phylogenetic analyses. We also present data on the timing, level, and location of expression of the genes as well as protein–protein interactions of them. These results suggest that subfunctionalization does act as a main mechanism for the survival of multiple gene copies in *A. trifoliata*.

Materials and methods

Plant materials

A. trifoliata plants were grown in the Botanical Garden, Institute of Botany, Chinese Academy of Sciences, Beijing. Inflorescences, floral buds at different developmental stages, and leaves were harvested and stored in the liquid

nitrogen for RNA isolation or embedded in paraplast for in situ hybridization.

Scanning electron microscopy

Inflorescences and floral buds were collected at 7-d intervals and immediately fixed with FAA (formalin to acetic acid to 50% ethanol=5:6:89). The specimens were dissected under a binocular microscope. Then, appropriate dissections were dehydrated in an ethanol–isoamyl acetate series, critical point dried in CO_2, coated with gold, and photographed using a Hitachi S-800 scanning electron microscope.

Isolation of APETALA3 and PISTILLATA homologs

Total RNA of inflorescences and flower buds was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Poly (A) mRNA was purified from total RNA using Oligotex mRNA Mini kit (Qiagen, Hilden, Germany). First strand cDNA was synthesized by SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with Poly (T) primer PT. Hemi-nested PCR amplification was performed with the degenerate primer p5B and the adapter primer PTAP. The amplified fragments over 800 bp were cloned into pGEM T easy vector (Promega, Madison, WI, USA). One hundred and nineteen clones were characterized by restriction analysis and/or sequencing. For each candidate locus, 8–16 clones were sequenced. The complete cDNA sequences were obtained by 5′ RACE. The sequences of the primers used in 5′ RACE procedure were p3AP31 and p3AP32 for AP3 homologs and p3PI1, p3PI2, and p3PI3 for PI homologs. Primer sequences are listed in Table S1.

Sequence alignments and phylogenetic analyses

All the genes obtained in this study and their homologs in other basal eudicots retrieved from NCBI database were used for phylogenetic analyses. If several sequences identified from one species showed the identity above 95% and contain no indels, they were looked as alleles and excluded (Stellari et al. 2004). First, full-length amino acid sequences of *AP3*- and *PI*-like genes were aligned with CLUSTALX 1.83 (Thompson et al. 1997) under the default value, respectively. Then, the alignments were reevaluated based on comparisons of closely related sequences and adjusted using Genedoc software by hand (Nicholas and Nicholas 1997). The corresponding DNA matrices were generated by aa2dna on the basis of the well-aligned protein matrices (http://www.bio.psu.edu/People/Faculty/Nei/Lab/software.htm). Phylogenetic analyses were performed based on the full-length DNA sequences of the two matrices.

For the DNA matrices, maximum-likelihood (ML) and Bayesian inference methods were used. Modeltest version 3.06 (Posada and Crandall 1998) was used to select the best fit model of molecular evolution. ML analysis was carried out using PHYML version 2.4.3 (Guindon and Gascuel 2003). The GTR + I + Γ model was chosen and ML parameter values were estimated to optimize. One thousand bootstrap replicates were performed (Felsenstein 1985) and a BIONJ tree was used as a starting point (Gascuel 1997). Bayesian analysis was performed using MrBayes version 3.0b4 (Ronquist and Huelsenbeck 2003). Four chains of Markov chain Monte Carlo were run, sampling one tree every 1,000 generations for 1,000,000 generations starting with a random tree. The first 50,000 generations were excluded as burn-in to ensure that the chains reached stationarity. The posterior probability was used to estimate nodal robustness.

Reverse transcriptase polymerase chain reaction analysis

Extraction of total RNAs, purification of poly (A) mRNAs, and synthesis of the first strand cDNAs were done according to the methods as described above. The template amount was regulated to be uniform using the actin gene. Then, gene-specific forward and reverse primers were used to detect the gene expression profiles. To make the results more convincing, the reverse transcriptase polymerase chain reaction (RT-PCR) was performed with 31, 28, 25, and 22 cycles. The annealing temperature was set depending on the T_m value of primer pairs, from 60 to 65°C. PCR products were fractionated in a 1.2% agarose gel and digitally photographed.

In situ hybridization

Inflorescences with floral primordia or immature flowers enclosed by 1–2 bracts and mature flower buds at various developmental stages were collected. The partial C-terminus and 3′ untranslated region of *AktAP3_1*(518–856), *AktAP3_2*(546–909), *AktAP3_3* (486–895), and *AktPI* (525–862) were used as templates for synthesizing sense or antisense digoxigenin-labeled RNA probes with DIG Northern starter kit (Roche Diagnostics, Mannheim, Germany). Embedding of plant materials, pretreatment, hybridization, and washing of the sections (8 μm) were performed as previously described (Li et al. 2005), except that final washing of the hybridized sections was carried out in 0.1×SSC at 55°C for 30 min.

Yeast two-hybrid assay

PCR products containing the full-length coding region of *AktAP3_1*, *AktAP3_2*, *AktAP3_3*, and *AktPI* with *Nco*I recognition site at the start codon were digested with *Nco*I

and another proper restriction enzyme and were introduced into the activation-domain (AD) vector pACT2 or the DNA-binding-domain (BD) vector pAS2 to yield the constructs of pACT2-AktAP3_1, pACT2-AktAP3_2, pACT2-AktAP3_3, pACT2-AktPI, pAS2-AktAP3_1, pAS2-AktAP3_2, pAS2-AktAP3_3, and pAS2-AktPI. The yeast strain AH109 was transformed with above constructs according to the manufacture's protocol of small-scale LiAc yeast transformation procedure. For autoactivation test, the single transformants were checked for *His3* reporter gene expression by incubation on appropriate synthetic dropout (SD) medium lacking histidine and leucine or tryptophan with 5 mM 3-AT (3-amino-1, 2, 4-trizole) (SD-His-Leu +5 mM 3-AT or SD-His-Trp +5 mM 3-AT). As a result, there was no colony grown on the corresponding selective medium, indicating that these transformants could be used further two-hybrid assays. The pACT2-AktAP3_1, pACT2-AktAP3_2, pACT2-AktAP3_3, and pACT2-AktPI transformants were used for subsequential transformations of each of pAS2 constructs. The pACT2 transformant was used to transform each pAS2 construct and the pAS2 transformant was used to transform each pACT2 construct, which were as negative controls. All transformants were plated on the SD medium (SD–Leu–Trp). Then, the positive colonies were diluted serially and cultured on the SD selective medium (SD–Leu–Trp–His–Adenine +5 mM 3-AT) for *His* and *Ade* reporter gene expression test, or checked the *LacZ* reporter gene expression by X-Gal with colony-lift filter method. All combinations in the negative controls did not grow in the selective medium.

Results

Morphology and ontogeny of inflorescence and flowers in *A. trifoliata*

The inflorescence of *A. trifoliata* consists of one to three female flowers at the base and numerous male flowers in the upper part (Fig. 1a). The female flower is dark red or purple, and has three petaloid sepals, six sterile staminodes, and six to nine carpels, which are open and conduplicate (Fig. 1b). The male flower, which is only faintly reddish and much smaller than the female flower, also has three petaloid sepals, and six stamens incurving over the 3–6 rudimentary carpels (Fig. 1c). Both male and female flowers have no petals (Fig. 1).

Using scanning electron microscopy (SEM), we investigated the initiation and development of the *A. trifoliata* flowers. To facilitate description, we arbitrarily divided the development of the flower into six stages. At stage 1 (s1), a number of spherical floral primordia arrange spirally around the inflorescence axis (Fig. S1a). At s2, sepals

Fig. 1 Morphology of *A. trifoliata* flowers. **a** Inflorescence including two female flowers at the base and many male flowers in the upper part. **b** Female flower. **c** Male flower

begin to initiate, and the floral apex becomes suborbicular or triangular and slightly convex at the center (Fig. S1b,d,e; Fig. S2a,b). When the sepals develop from triangular to suborbicular (s3, Fig. S1c), six stamen primordia begin to initiate (s3, Fig. S1f). No petal primordia were observed between the sepal and stamen primordia. During s4–5, the stamen primordia differentiate and then form anthers (Fig. S1g–j; Fig. S2c–e). In the meantime, the carpel primordia initiate spirally at s4 (Fig. S1g; Fig. S2c) and subsequently elongate upward and grow into cylinder (Fig. S1h, i; Fig. S2d). After that, the lateral parts of the carpel develop quickly (s5, Fig. S1j; Fig. S2e), gradually incurve to the center (s6, Fig. S1k, l; Fig. S2f). Finally, open and conduplicate carpels are established (s6, Fig. S1k, l; Fig. S2f).

From s1 to s5, the female and male flowers are almost indistinguishable. But at s6, we can distinguish them because the carpels in the male flower and the stamens in the female flower begin to cease developing. As a result, in the male flower, the carpels are smaller and surrounded by the stamens (s6, Fig. S1k). In contrast, the stamens in the female flower become inconspicuous due to the quick growth of the carpels (s6, Fig. S2f).

Phylogenetic analyses of class B genes in *A. trifoliata*

We identified cDNAs of four different putative class B MADS-box genes from over one hundred clones. Three of them represented paleo*AP3*-like genes and one a *PI*-like gene. BLAST search in GenBank demonstrated that they were highly similar to the class B genes from *Akebia quinata* (Kramer et al. 2003). The four genes were designated *AktAP3_1* (*Akebia trifoliata APETALA3_1*, AY627630), *AktAP3_2* (AY627632), *AktAP3_3* (DQ303124), and *AktPI* (*Akebia trifoliata PISTILLATA*, AY627634), respectively.

Based on the phylogenetic tree of *AP3*-like genes, *AktAP3_1* is orthologous to *AkqAP3-2* of *A. quinata*, while *AktAP3_2* and *AktAP3_3* correspond to *AkqAP3-1type 1* and *AkqAP3-1type 2*, respectively. They were produced by two recent duplication events. One giving rise to *AktAP3_2* and *AktAP3_3* occurred before the origin of the genus *Akebia*; while the exact timing and placement of the other producing *AktAP3_1* and *AktAP3_2/3* is difficult to be determined based on the current sampling (Fig. 2a). In the study of Kramer et al. (2003), *AkqAP3-1type 1* and *AkqAP3-1type 2* were interpreted as being allelic. In this study, we obtained their apparent orthologs from *A. trifoliata*. Moreover, the protein sequences of *AktAP3_2* and *AkqAP3-1type 1* show 98% identities, and those of *AktAP3_3* and *AkqAP3-1* type 2 are 96% identical. In contrast, *AktAP3_2* and *AktAP3_3* are 94% identical to each other at the protein level, and some interesting differences were observed by sequence comparison: (1) they contain several gene-specific indels throughout the coding region; (2) they show obvious sequence divergence of about 100 nucleotides in the 3′-UTR. The same phenomenon is observed for *AkqAP3-1type 1* and *AkqAP3-1type 2* (Kramer et al. 2003). Based on these facts, we believe that *AktAP3_2* and *AktAP3_3* are from very closely related, but distinctively different loci. Most species of the Ranunculaceae generally also have three types of *AP3*-like genes, but they are produced by additional duplications, which are independent of those events for producing *Akebia*, *AP3*-like genes (Fig. 2a). In the case of *AktPI*, it is highly similar to *AkqPI*. As indicated in the study of Kramer et al. (2003), the *PI* homologs in Ranunculales appear to be produced by many recent duplication events. However, no duplications are observed in *A. trifoliata* and *A. quinata* based on the current phylogenetic tree, because only one *PI*-like gene is identified in each species so far (Fig. 2b). Taken together, in the phylogenetic trees reconstructed by *AP3*- and *PI*-like genes, none of the genes from *Akebia* grouped with the homologs of the Ranunculaceae and Berberidaceae (Fig. 2). Therefore, the class B genes from different plant families in Ranunculales very likely experienced different evolutionary histories.

Temporal and spatial expression patterns of the class B genes in *A. trifoliata*

To determine the expression patterns of the putative class B genes from *A. trifoliata*, we performed RT-PCR with male flowers, female flowers, inflorescence axes, and leaves. We found that all these four genes are mainly expressed in male and female flowers, but at different levels (Fig. 3). *AktAP3_1* is expressed more strongly in female flowers than in male flowers (Fig. 3a). In contrast, *AktAP3_2* is expressed at higher levels in male flowers than in female flowers (Fig. 3b). The expression levels of *AktAP3_3* and *AktPI* in male or female flowers are similar (Fig. 3c,d). Furthermore, *AktAP3_1* is expressed at relatively high levels in inflorescence axes (Fig. 3a), whereas *AktAP3_3* and *AktPI* are expressed there at lower levels (Fig. 3c,d). *AktAP3_2* is not expressed in inflorescence axes (Fig. 3b). The expression signals of these genes are almost undetectable in leaves except that *AktAP3_1* shows a trace of expression (Fig. 3a).

To further investigate the spatial and temporal expression patterns of the genes, in situ hybridizations were performed at a series of developmental stages of floral buds (s2, s4, and s6). In s2, *AktAP3_1* and *AktAP3_3* is expressed in the androecial and gynoecial primordia [Fig. 4a (1, 2), c (1, 2)]; while *AktAP3_2* is mainly restricted to the adaxial parts of the androecial primordia [Fig. 4b (1, 2)]. Moreover, weak expression of *AktAP3_3* was observed in the sepal whorl [Fig. 4c (1, 2)]. *AktPI* mRNA was found in the androecial primordia and the developing sepals [Fig. 4d (1, 2)]. After the initiation of the gynoecial primordia (s4), transcripts of the four class B genes are accumulated in the developing stamens and carpels [Fig. 4a (3), b (3), c (3), d (3)]. After the flowers enter unisexual development at stage 6, all the four genes are expressed in the pollen sacs of the stamens and the rudimentary carpels of the male flower [Fig. 4a (4, 5), b (4, 5), c (4, 5), d (4, 5)]. The transcripts of these genes were also observed in the staminodes of the female flower [Fig. 4a (6), b (6), c (6), d (6)]. However, only *AktAP3_1* and *AktPI* were detected in the carpels of the female flower [Fig. 4a (6), d (6)].

Interaction patterns of class B proteins in *A. trifoliata*

Although the sequence alignment showed that the AktAP3_1, AktAP3_2, and AktAP3_3 proteins share very similar primary structures of protein, they display different interaction patterns with AktPI in the yeast two-hybrid assay. As shown in Fig. 5, AktAP3_1 is able to interact strongly with AktPI (Fig. 5d,m), AktAP3_2 with AktPI at medium strength (Fig. 5h,n), while AktAP3_3 heterodimerizes more weakly with AktPI (Fig. 5l,o), no matter whether the AktAP3_1/2/3 are transformed as DNA-binding-domain or activation-domain vectors. Moreover, AktAP3_1, AktAP3_2, and AktAP3_3 could neither form homodimers (Fig. 5a,f,k), nor interact with the other AP3-like proteins (Fig. 5b,c,e,g,i,j). AktPI is not able to form homodimers (Fig. 5p).

Discussion

Class B genes display broader expression in basal eudicots than in core eudicots

In our study, cDNAs of four putative class B genes were isolated from *A. trifoliata*. In situ hybridization analyses show that these genes are initially expressed in the stamen

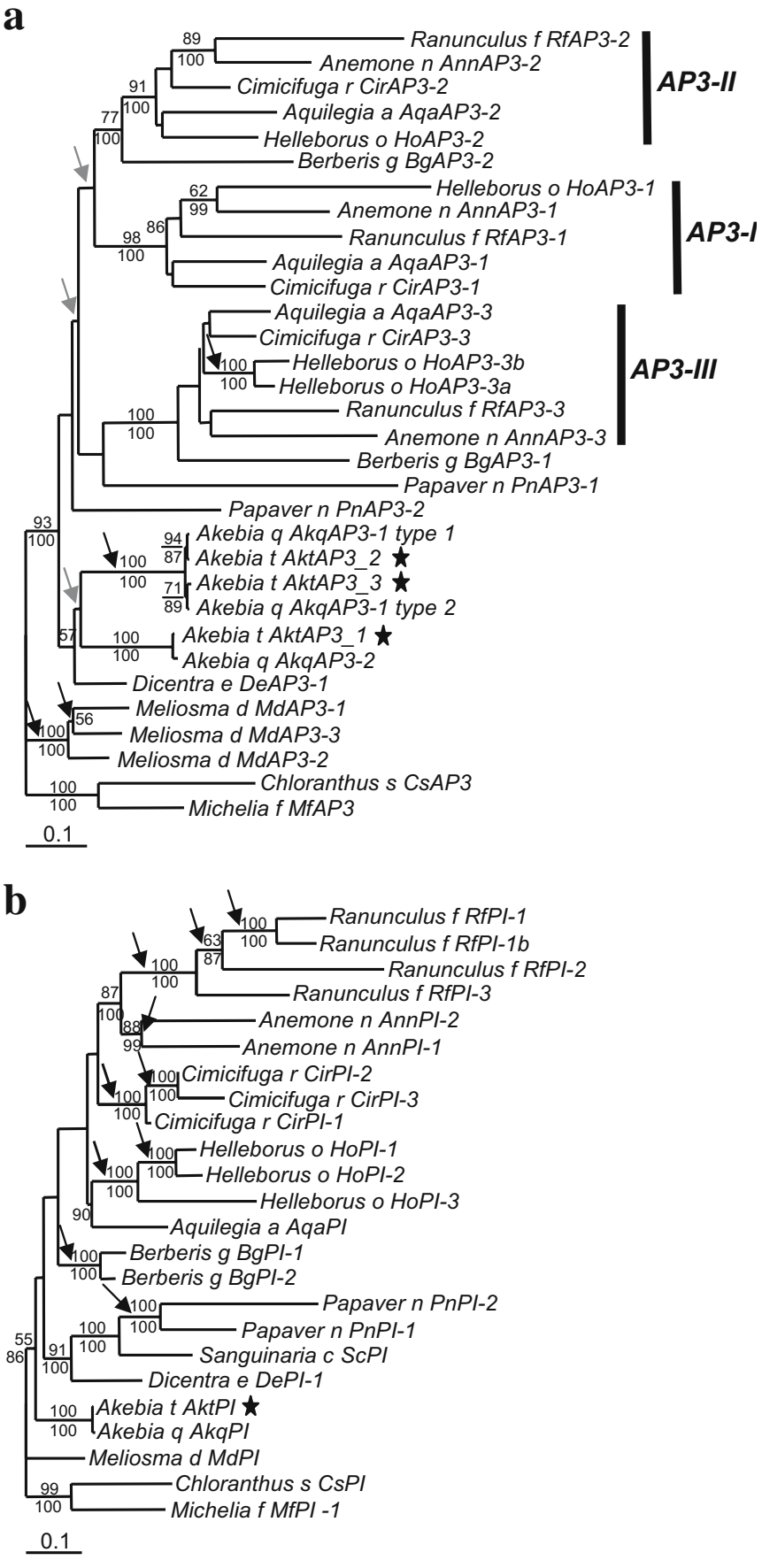

Fig. 2 Maximum-likelihood tree of *AP3*- (**a**) and *PI*-like (**b**) genes based on the nucleotide matrix including full-length sequences. The *numbers above branch* are bootstrap values (>50%) from 1,000 replicates in PHYML analysis. The *values below branch* are posterior probabilities (>70) from Bayesian analysis. *Asterisks* indicate genes identified in this study. The *black arrow* indicates inferred duplication event. The *gray arrow* indicates that the timing and placement of the duplication event are not certain. **a** The *AP3*-like genes from the Ranunculaceae form three clades, represented by *AP3-I*, *AP3-II*, and *AP3-III*. The tree was rooted with *MfAP3* from *Michelia figo* and *CsAP3* from *Chloranthus spicatus*. **b** The tree was rooted with *MfPI-1* from *Michelia figo* and *CsPI* from *Chloranthus spicatus*

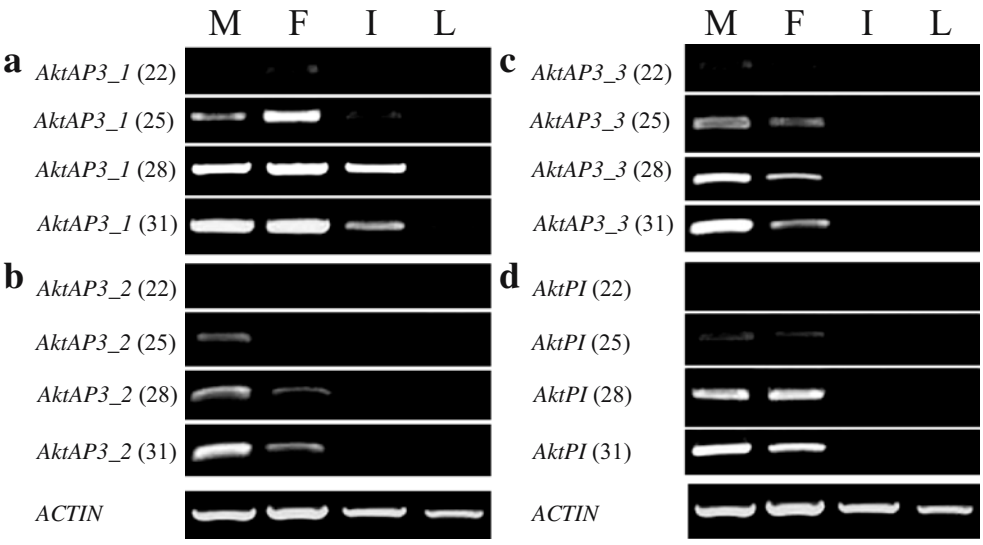

Fig. 3 RT-PCR results of *AktAP3_1*, *AktAP3_2*, *AktAP3_3*, and *AktPI*. RNAs were isolated from male flower (*M*), female flower (*F*), inflorescence axis (*I*), and leaves (*L*) of *A. trifoliata* and RT-PCR were performed with gene-specific primers. The PCR cycle numbers are indicated in the parentheses

primordia. As floral organs develop, accumulation of their transcripts is increased in the regions of the developing stamens. These results suggest that the class B genes in *A. trifoliata* function in specifying stamen identity. This is consistent with previous functional and expression analyses of class B genes from other angiosperm species (e.g., Goto and Meyerowitz 1994; Jack et al. 1992; Kim et al. 2005a,b; Kramer and Irish 1999, 2000; Lamb and Irish 2003; Sommer et al. 1990; Trobner et al. 1992; Vandenbussche et al. 2004). Therefore, our observations suggest that the ancestral role of defining male reproductive organs (Sundström and Engström 2002; Winter et al. 1999, 2002b) has been retained regardless of the multiple (at least two) duplication events that occurred in the class B gene subfamily and the changes in the C-terminus of the *AP3* gene lineage during the evolution of the angiosperms.

Besides the conserved expression in the stamens, the four class B genes are also expressed in the developing carpel of *A. trifoliata*. Actually, the paleo*AP3*- (including *TM6*-) and *PI*-like genes are expressed in carpels in many angiosperm species, especially in basal angiosperm and basal eudicot species (Kim et al. 2005a,b; Kramer and Irish 2000; Kramer et al. 2003; Munster et al. 2001). The latest study on expression in the probably basalmost angiosperms *Amborella trichopoda* and *Nuphar advena* suggested that the paleo*AP3* and *PI* homologs are expressed in the perianth, stamen and carpel (Kim et al. 2005b). The paleo*AP3*- and *PI*-like genes from *Eupomatia bennettii*, a basal angiosperm species, also exhibit a broader expression in the calyptra, stamen, and carpel (Kim et al. 2005a). Similar expression patterns could be observed in basal eudicot species, such as *Papaver nudicaule*, *Sangunaria canadensis*, *Dicentra eximia*, *Ranunculus ficaria*, and others (Kramer and Irish 2000; Kramer et al. 2003). The broader expression domains of paleo*AP3* and *PI* genes in the basal angiosperms and basal eudicots are obviously different from that of class B genes in gymnosperms, where expression is strictly confined to the male reproductive organs (Sundström and Engström 2002; Winter et al. 1999, 2002b). It is possible that because of the duplication of the ancestral class B genes before the occurrence of extant angiosperms (Aoki et al. 2004; Kim et al. 2004b; Kramer et al. 1998), the novel duplicated paleo*AP3* and *PI* lineage might have evolved under relaxed selection pressure (Lynch and Conery 2000) and obtained broader expression ranges. However, there is no direct evidence so far, such as mutant phenotypes, that the class B genes in the basal angiosperms and basal eudicots are involved in specifying carpel identity.

Previous morphological description suggested that the mature flower of *A. trifoliata* has one whorl of petaloid perianth (Qin 1997). Moreover, our SEM investigation demonstrated that the perianth primordia look like sepal primordia, because employing SEM, similar sepal primordia were observed in the flowers of *Sinofranchetia chinensis*, which belongs to the same plant family but has differentiated sepals and petals (Zhang et al., unpublished data). Therefore, we think that the colorful petaloid perianth is made of sepals, rather than petals. Generally, the feature of petaloid organs is correlated with the expression of class B genes, regardless of their position. This has been supported by the expressions of *AP3*/*PI* homologs in petaloid perianth of many monocot species, such as *Tulipa gesneriana* (Kanno et al. 2003), *Lilium regale* (Theissen et al. 2000), and *Agapanthus praecox* ssp. *orientalis* (Nakamura et al. 2005) and several basal eudicot species (Kramer and Irish 2000; Kramer et al. 2003). In *A. trifoliata*, two class B genes, *AktAP3_3* and *AktPI*, are expressed in the developing petaloid sepals at

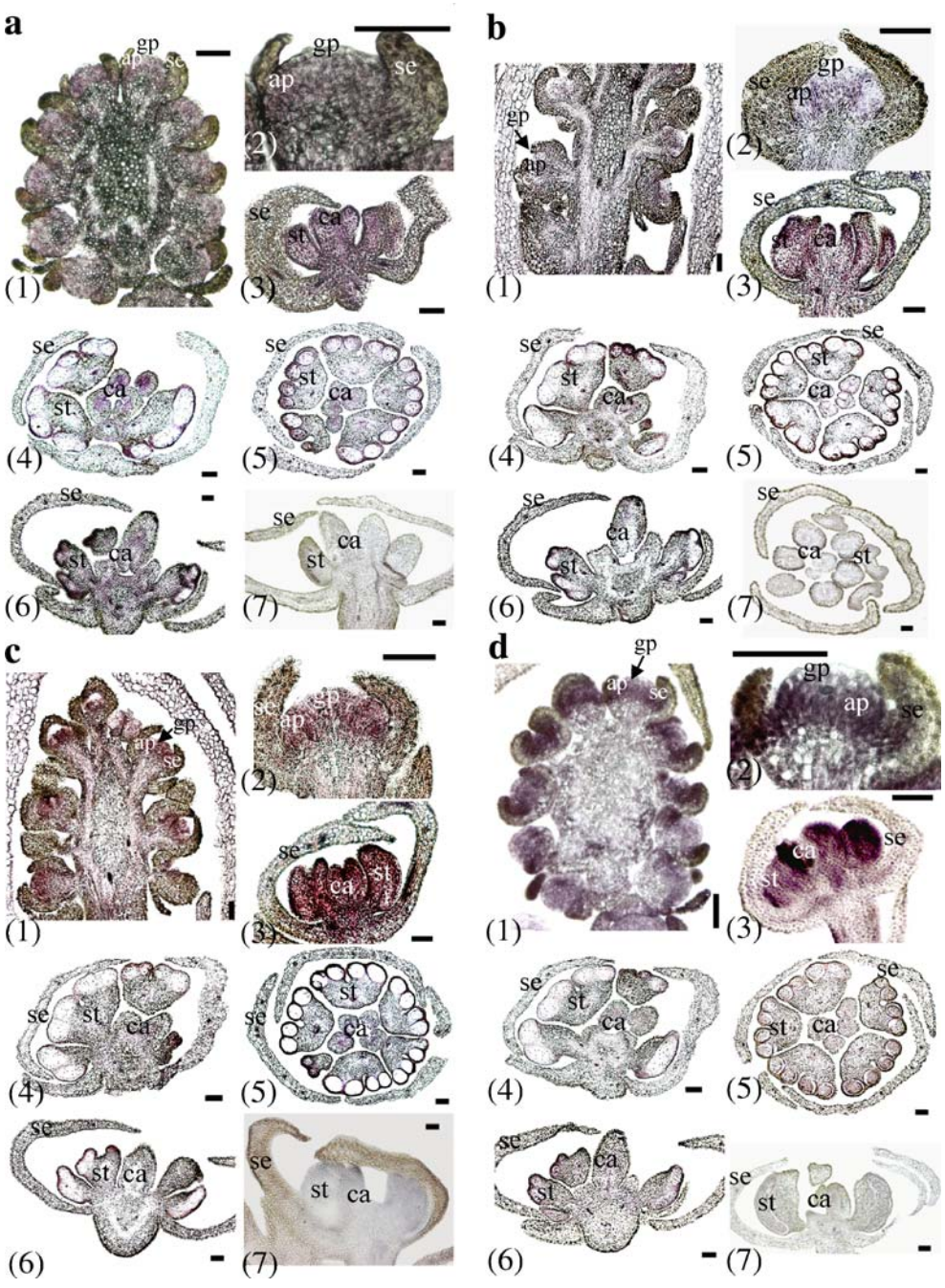

Fig. 4 In situ expression of *AktAP3_1* (**a**), *AktAP3_2* (**b**), *AktAP3_3* (**c**), and *AktPI* (**d**). (*1*)–(*6*) Antisense probe hybridization for each gene. (*1*) Inflorescence with early floral buds. (*2*) Early floral bud with androecial and gynoecial primordia (s2). (*3*) Floral bud with developing stamens and carpels (s4). (*4*)–(*6*) Male and female flowers at s6. (*4*) Male flower with rudimentary carpels, longitudinal section. (*5*) Male flower, transverse section. (*6*) Female flower with staminodes, longitudinal section. (*7*) Negative control of sense probe for each gene. *ap* Androecial primordia; *gp* gynoecial primordia; *se* sepal; *st* stamen; *ca* carpel. *Scale bars* 100 μm. The *pink* or *purple* color represents the hybridization signal

earlier stages of flower development. Our results are in agreement with the expression patterns of class B genes in above species and imply that the petaloid character of the sepals might be regulated by the class B genes.

Class B proteins of *A. trifoliata* constitute obligate heterodimers

In this study, we explored the protein interaction mode in basal eudicots by using yeast two-hybrid. In *A. trifoliata*, the three paralogous AP3 proteins do not act as homodimers, but require AktPI as a partner in vitro. Furthermore, AktPI cannot form homodimers and the three AktAP3 proteins are not able to heterodimerize. The *AP3* genes from *A. trifoliata* belong to the paleo*AP3* lineage. Although the expression patterns of these genes are similar to those observed on the class B genes from other basal eudicot and basal angiosperm species, the interaction patterns resemble those in the core eudicots. Outside the core eudicots, it is poorly known about the interaction modes of class B proteins except for some monocot species of basal angiosperms. For instance, in *Lilium regale*, LRDEF, a paleoAP3-like protein, can form heterodimers with LRGLOA or LRGLOB that can also form homodimers

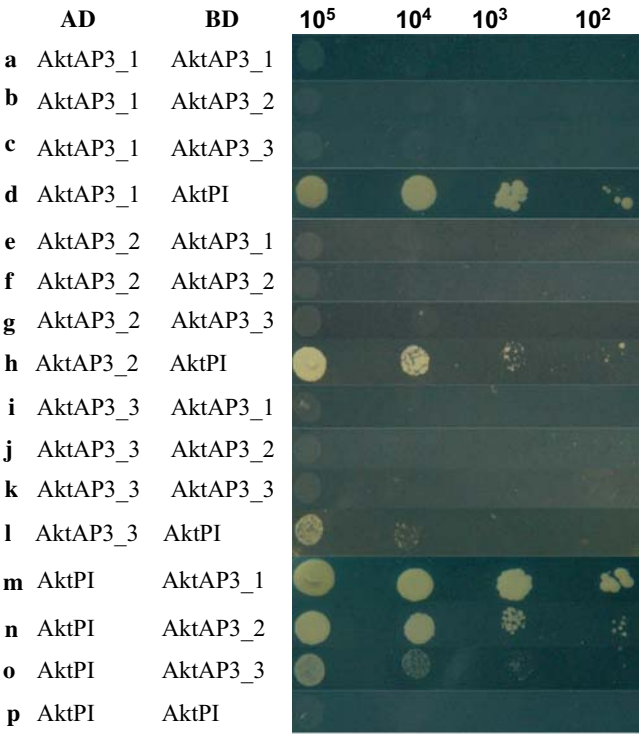

Fig. 5 Interactions of class B MADS-domain proteins in *A. trifoliata*. Serial dilutions of 10^5–10^2 AH109 cells containing different plasmid combinations were grown on the selective medium SD-LTHA + 5 mM 3-AT. *L* Leucine; *T* tryptophan; *H* histidine; *A* adenine; *3-AT* 3-amino-1,2,4-triazole

(Winter et al. 2002a). In *Tulipa gesneriana*, two DEF-like proteins, TGDEFA and TGDEFB, cannot homodimerize, but both can interact with a GLO-like protein TGGLO that is able to form homodimers like the GLO-like proteins in *L. regale* (Kanno et al. 2003). However, the paleoAP3-like protein OMADS3 from *Oncidium* Gower Ramsey and LMADS1 from *Lilium longiflorum* can form homodimers (Hsu and Yang 2002; Tzeng et al. 2004). These data are inconsistent with the observations in rice and maize. The paleoAP3 orthologs OsAMDS16 from rice and SILKY1 from maize only form obligate heterodimers with their PI partners OsMADS4 or ZMM16, respectively, which are not capable of forming homodimers (Moon et al. 1999; Whipple et al. 2004). Therefore, on the basis of all the available information regarding the interaction patterns of class B proteins, we speculate that multiple origins of the obligate heterodimerization between AP3- and PI-like proteins very likely have occurred throughout the evolution of the flowering plants.

Functional divergence of duplicated AP3 genes and its evolutionary implication

In *A. trifoliata*, the expression patterns of the three duplicated *AktAP3* genes have obviously diverged. *AktAP3_1* is strongly expressed in female flowers and *AktAP3_2* is mostly transcribed in male flowers. *AktAP3_3* is expressed in both female and male flowers at similar levels. Moreover, at the earlier stages of flower development, the three *AP3*-like paralogs display different spatial expression profiles. After gene duplication, in addition to the divergence of expression patterns, the paralogous transcription factors might obtain different affinities with their partners. For instance, in *Petunia hybrida*, there is evidence that PHTM6 selectively interacts with the PI-like proteins, PHGLO1, and PHGLO2 (Vandenbussche et al. 2004). In this study, similar situation was observed in a basal eudicot species, *A. trifoliata*. By yeast two-hybrid analyses, we found that the AktAP3_1/2/3 proteins are able to form heterodimers with the AktPI, but with different strength. Combining the results of expression analyses, we speculate that AktAP3_1/AktPI predominantly functions in the development of female flowers and AktAP3_2/AktPI mainly in the male flowers; *AktAP3_1* and *AktAP3_2* might be differentially regulated in the male and female flowers of *A. trifoliata*. Although *AktAP3_3* and *AktPI* show overlapping expression patterns in stamens and developing petaloid sepals, they only interact fairly weakly in vitro. Based on our findings, it appears likely that in *A. trifoliata*, these three paralogous *AP3*-like genes have diverged in the expression patterns and interaction behaviors, and collectively fulfill the functions of the ancestral *AP3* gene. This would represent a classical case of subfunctionalization (Force et al. 1999; Lynch and Force 2000).

However, the divergence of expression and interaction patterns of the duplicated *AP3* genes in *A. trifoliata* may only reflect one of various situations in the basal eudicots. Because of the diverse architectures of flowers, basal eudicot families were traditionally included into different subclasses in several classification systems of angiosperms (e.g., Cronquist 1988; Takhtajan 1997). And the phylogenetic relationships among basal eudicot families are still controversial based on the molecular phylogenetic analyses. In addition, many independent gene duplications in the *DEF*/*GLO* subfamily have been revealed in Ranunculales, (Kramer et al. 2003; this study). Therefore, as the transitional group between basal angiosperms and core eudicots, different basal eudicot lineages might have acquired different interaction patterns among the multiple AP3 and PI proteins, which not only may have contributed to the floral diversity of basal eudicots, but also may have provided sufficient genetic resources for the "explosive" radiation of core eudicots and the fixation of differentiated perianth morphology in the core eudicots. Clearly, in a next step, more basal eudicot species have to be studied to better understand the diversity and evolution of the angiosperm flower.

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References

Albert VA, Gustafsson MHG, DiLaurenzio L (1998) Ontogenetic systematics, molecular developmental genetics, and the angiosperm petal. In: Soltis DE, Soltis PS, Doyle JJ (eds) Molecular systematics of plant II. Kluwer, Boston, pp 349–374

Aoki S, Uehara K, Imafuku M, Hasebe M, Ito M (2004) Phylogeny and divergence of basal angiosperms inferred from *APETALA3*- and *PISTILLATA*-like MADS-box genes. J Plant Res 117:229–244

APG (The Angiosperm Phylogeny Group) (2003) An update of the angiosperm phylogeny group classification for the orders and families of flowering plants: APG II. Bot J Linn Soc 141: 399–436

Becker A, Theissen G (2003) The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Mol Phylogenet Evol 29:464–489

Coen ES, Meyerowitz EM (1991) The war of the whorls: genetic interactions controlling flower development. Nature 353:31–37

Cronquist A (1988) The evolution and classification of flowering plants. Columbia University Press, New York

De Craene LPR, Soltis PS, Soltis DE (2003) Evolution of floral structures in basal angiosperms. Int J Plant Sci 164:S329– S363

Di Stilio VS, Kramer EM, Baum DA (2005) Floral MADS box genes and homeotic gender dimorphism in *Thalictrum dioicum* (Ranunculaceae)—a new model for the study of dioecy. Plant J 41:755–766

Drinnan AN, Crane PR, Hoot SB (1994) Patterns of floral evolution in the early diversification of non-magnoliid dicotyledons (eudicots). Plant Syst Evol 8(Suppl):93–122

Duarte JM, Cui L, Wall PK, Zhang Q, Zhang X, Leebens-Mack J, Ma H, Altman N, dePamphilis CW (2006) Expression pattern shifts following duplication indicative of subfunctionalization and neofunctionalization in regulatory genes of *Arabidopsis*. Mol Biol Evol 23:469–478

Endress PK (1992) Evolution and floral diversity: the phylogenetic surroundings of *Arabidopsis* and *Antirrhinum*. Int J Plant Sci 153:S106–S122

Endress PK (1994) Floral structure and evolution of primitive angiosperms: recent advances. Plant Syst Evol 192:79–97

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Force A, Lynch M, Pickett FB, Amores A, Yan YL, Postlethwait J (1999) Preservation of duplicate genes by complementary, degenerative mutations. Genetics 151:1531–1545

Gascuel O (1997) BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. Mol Biol Evol 14: 685–695

Goto K, Meyerowitz EM (1994) Function and regulation of the *Arabidopsis* floral homeotic gene *PISTILLATA*. Genes Dev 8:1548–1560

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:596–704

Hoot SB, Culham A, Crane PR (1995) The utility of *atpB* gene sequences in resolving phylogenetic relationships: comparison with *rbcL* and 18S ribosomal DNA sequences in the Lardizabalaceae. Ann Mo Bot Gard 82:194–207

Hoot SB, Magallón S, Crane PR (1999) Phylogeny of basal eudicots based on three molecular data sets: *atp*B, *rbc*L, and 18S nuclear ribosomal DNA sequences. Ann Mo Bot Gard 86:1–32

Hsu HF, Yang CH (2002) An Orchid (*Oncidium* Gower Ramsey) *AP3*-like MADS gene regulates floral formation and initiation. Plant Cell Physiol 43:1198–1209

Irish VF (2003) The evolution of floral homeotic gene function. Bioessays 25:637–646

Jack T, Brockman LL, Meyerowitz EM (1992) The homeotic gene *APETALA3* of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. Cell 68:683–697

Kanno A, Saeki H, Kameya T, Saedler H, Theissen G (2003) Heterotopic expression of class B floral homeotic genes supports a modified ABC model for tulip (*Tulipa gesneriana*). Plant Mol Biol 52:831–841

Kaufmann K, Melzer R, Theissen G (2005) MIKC-type MADS-domain proteins: structural modularity, protein interactions and network evolution in land plants. Gene 347:183–198

Kim S, Soltis DE, Soltis PS, Zanis MJ, Suh Y (2004a) Phylogenetic relationships among early-diverging eudicots based on four genes: were the eudicots ancestrally woody? Mol Phylogenet Evol 31:16–30

Kim S, Yoo MJ, Albert VA, Farris JS, Soltis PS, Soltis DE (2004b) Phylogeny and diversification of B-function MADS-box genes in angiosperms: evolutionary and functional implications of a 260-million-year-old duplication. Am J Bot 91:2102–2118

Kim S, Koh J, Ma H, Hu Y, Endress PK, Hauser BA, Buzgo M, Soltis PS, Soltis DE (2005a) Sequence and expression studies of A-, B-, and E-class MADS-box homologues in *Eupomatia* (Eupomatiaceae): support for the bracteate origin of the calyptra. Int J Plant Sci 166:185–198

Kim S, Koh J, Yoo MJ, Kong H, Hu Y, Ma H, Soltis PS, Soltis DE (2005b) Expression of floral MADS-box genes in basal angiosperms: implications for the evolution of floral regulators. Plant J 43:724–744

Kramer EM, Dorit RL, Irish VF (1998) Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the *APETALA3* and *PISTILLATA* MADS-box gene lineages. Genetics 149:765–783

Kramer EM, Irish VF (1999) Evolution of genetic mechanisms controlling petal development. Nature 399:144–148

Kramer EM, Irish VF (2000) Evolution of the petal and stamen developmental programs: evidence from comparative studies of the lower eudicots and basal angiosperms. Int J Plant Sci 161:S29–S40

Kramer EM, Di Stilio VS, Schluter PM (2003) Complex patterns of gene duplication in the *APETALA3* and *PISTILLATA* lineages of the Ranunculaceae. Int J Plant Sci 164:1–11

Lamb RS, Irish VF (2003) Functional divergence within the *APETALA3*/*PISTILLATA* floral homeotic gene lineages. Proc Natl Acad Sci USA 100:6558–6563

Li GS, Meng Z, Kong HZ, Chen ZD, Theissen G, Lu AM (2005) Characterization of candidate class A, B and E floral homeotic genes from the perianthless basal angiosperm *Chloranthus spicatus* (Chloranthaceae). Dev Genes Evol 215:437–449

Litt A, Irish VF (2003) Duplication and diversification in the *APETALA1*/*FRUITFULL* floral homeotic gene lineage: implications for the evolution of floral development. Genetics 165: 821–833

Lynch M, Conery JS (2000) The evolutionary fate and consequences of duplicate genes. Science 290:1151–1155

Lynch M, Force A (2000) The probability of duplicate gene preservation by subfunctionalization. Genetics 154:459–473

Ma H, dePamphilis C (2000) The ABCs of floral evolution. Cell 101:5–8

Matsunaga S, Isono E, Kejnovsky E, Vyskot B, Dolezel J, Kawano S, Charlesworth D (2003) Duplicative transfer of a MADS box gene to a plant Y chromosome. Mol Biol Evol 20:1062–1069

Moon YH, Jung JY, Kang HG, An G (1999) Identification of a rice *APETALA3* homologue by yeast two-hybrid screening. Plant Mol Biol 40:167–177

Munster T, Wingen LU, Faigl W, Werth S, Saedler H, Theissen G (2001) Characterization of three *GLOBOSA*-like MADS-box genes from maize: evidence for ancient paralogy in one class of floral homeotic B-function genes of grasses. Gene 262:1–13

Nakamura T, Fukuda T, Nakano M, Hasebe M, Kameya T, Kanno A (2005) The modified ABC model explains the development of the petaloid perianth of *Agapanthus praecox* ssp. *orientalis* (Agapanthaceae) flowers. Plant Mol Biol 58:435–445

Ng M, Yanofsky MF (2001) Function and evolution of the plant MADS-box gene family. Nat Rev Genet 2:186–195

Nicholas KB, Nicholas HB (1997) Genedoc: a tool for editing and annotating multiple sequence alignments. Distributed by the author

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–818

Qin HN (1997) A taxonomic revision of the lardizabalaceae. In: Cathaya 8–9. International Academic Publishers, Beijing

Riechmann JL, Krizek BA, Meyerowitz EM (1996) Dimerization specificity of *Arabidopsis* MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA, and AGAMOUS. Proc Natl Acad Sci USA 93:4793–4798

Riechmann JL, Meyerowitz EM (1997) MADS domain proteins in plant development. Biol Chem 378:1079–1101

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Schwarz-Sommer Z, Hue I, Huijser P, Flor PJ, Hansen R, Tetens F, Lonnig WE, Saedler H, Sommer H (1992) Characterization of the *Antirrhinum* floral homeotic MADS-box gene *deficiens*: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. EMBO J 11:251–263

Soltis DE, Senters A, Zanis MJ, Kim S, Thompson JD, Soltis PS, De Craene LPR, Endress PK, Farris JS (2003) Gunnerales are sister to other core eudicots: implications for the evolution of pentamery. Am J Bot 90:461–470

Sommer H, Beltran JP, Huijser P, Pape H, Lonnig WE, Saedler H, Schwarz-Sommer Z (1990) *DEFICIENS*, a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus*: the protein shows homology to transcription factors. EMBO J 9:605–613

Stellari GM, Jaramillo MA, Kramer EM (2004) Evolution of the *APETALA3* and *PISTILLATA* lineages of MADS-box-containing genes in the basal angiosperms. Mol Biol Evol 21:506–519

Sundström J, Engström P (2002) Conifer reproductive development involves B-type MADS-box genes with distinct and different activities in male organ primordia. Plant J 31:161–169

Takhtajan A (1997) Diversity and classification of flowering plants. Columbia University Press, New York

Theissen G, Becker A, Di Rosa A, Kanno A, Kim JT, Münster T, Winter KU, Saedler H (2000) A short history of MADS-box genes in plants. Plant Mol Biol 42:115–149

Theissen G, Kim JT, Saedler H (1996) Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes. J Mol Evol 43:484–516

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Trobner W, Ramirez L, Motte P, Hue I, Huijser P, Lonnig WE, Saedler H, Sommer H, Schwarz-Sommer Z (1992) *GLOBOSA*: a homeotic gene which interacts with *DEFICIENS* in the control of *Antirrhinum* floral organogenesis. EMBO J 11:4693–4704

Tzeng TY, Liu HC, Yang CH (2004) The C-terminal sequence of LMADS1 is essential for the formation of homodimers for B function proteins. J Biol Chem 279:10747–10755

Vandenbussche M, Theissen G, Van de Peer Y, Gerats T (2003) Structural diversification and neo-functionalization during floral MADS-box gene evolution by C-terminal frameshift mutations. Nucleic Acids Res 31:4401–4409

Vandenbussche M, Zethof J, Royaert S, Weterings K, Gerats T (2004) The duplicated B-class heterodimer model: whorl-specific effects and complex genetic interactions in Petunia hybrida flower development. Plant Cell 16:741–754

Van der Krol AR, Brunelle A, Tsuchimoto S, Chua NH (1993) Functional analysis of Petunia floral homeotic MADS box gene *pMADS1*. Genes Dev 7:1214–1228

Van der Krol AR, Chua NH (1993) Flower development in Petunia. Plant Cell 5:1195–1203

Whipple CJ, Ciceri P, Padilla CM, Ambrose BA, Bandong SL, Schmidt RJ (2004) Conservation of B-class floral homeotic gene function between maize and *Arabidopsis*. Development 131: 6083–6091

Winter KU, Becker A, Munster T, Kim JT, Saedler H, Theissen G (1999) MADS-box genes reveal that gnetophytes are more closely related to conifers than to flowering plants. Proc Natl Acad Sci USA 96:7342–7347

Winter KU, Weiser C, Kaufmann K, Bohne A, Kirchner C, Kanno A, Saedler H, Theissen G (2002a) Evolution of class B floral homeotic proteins: obligate heterodimerization originated from homodimerization. Mol Biol Evol 19:587–596

Winter KU, Saedler H, Theissen G (2002b) On the origin of class B floral homeotic genes: functional substitution and dominant inhibition in *Arabidopsis* by expression of an orthologue from the gymnosperm *Gnetum*. Plant J 31:457–475

Zahn LM, Leebens-Mack J, dePamphilis CW, Ma H, Theissen G (2005) To B or not to B a flower: the role of *DEFICIENS* and *GLOBOSA* orthologs in the evolution of the angiosperms. J Hered 96:225–240

Zanis MJ, Soltis PS, Qiu YL, Zimmer E, Soltis DE (2003) Phylogenetic analyses and perianth evolution in basal angiosperms. Ann Mo Bot Gard 90:129–150